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Transiently Transfected and Stably Integrated HIV-1 LTR Responds Differentially to the Silencing Activity of the Krüppel-Associated Box (KRAB) Transcriptional Repressor Domain

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It has been demonstrated previously that the transcriptional repressor domain called the Krüppel-associated box (KRAB), conserved in a large number of Krüppel-type zinc finger proteins, fused to Tat transdominant negative mutants, is able to silence HIV-1 long terminal repeat (LTR)-driven gene expression in transient transfection assays. In the present study chimeric Tat mutant-KRAB retroviral expression vectors were used to control HIV-1 replication in acutely infected cells. It was found that while transient and stable expression of Tat mutant-KRAB chimeric proteins represses HIV-1 LTR-driven gene transcription in transient assays, stable expression of Tat mutant-KRAB chimeric molecules does not confer resistance to HIV-1 infection in Jurkat T lymphocytic cell lines. The results provide further evidence that transient transfection may underestimate the role of chromosomal structure in transcriptional regulation and highlight the caveat of direct extrapolation of transient results for designing gene therapy strategies for efficient control of HIV-1 infection. ***J. Med. Virol. 58:264–272, 1999.***

KEY WORDS: HIV-1 Tat mutants; Krüppel-associated box; HIV-1 replication

INTRODUCTION

The Tat protein of HIV-1, located both in the intracellular and the extracellular compartment, is essential for HIV replication, is required for transition from latent to productive HIV-infection, and exerts pleiotropic activities on cellular functions through autocrine and paracrine mechanisms [Jones and Peterlin, 1994; Caputo et al., 1995; Chang et al., 1995]. Intracellular and extracellular Tat plays an important role in dysregulation of the immune system, in neurodegenerative processes, in reactivation of latent heterologous viral infections, and in the pathogenesis of tumors arising in the course of acquired immunodeficiency syndrome (AIDS), particularly in the development of Kaposi sarcoma (KS), the most frequent neoplastic complication in AIDS [Jones and Peterlin, 1994; Caputo et al., 1995; Chang et al., 1995]. Tat is an 86–102 amino acid (aa) protein encoded by two exons. Four functional domains have been identified in the first exon of Tat, called amino-terminal (aa 1–21), cysteine rich (aa 22–37), core (aa 38–48), and basic (aa 49–72). The cysteine-rich region represents the Tat transactivation domain. The basic region contains the nuclear localization signals and the binding sequence to the Tat-responsive element (TAR) located at the 5′ end of all viral RNAs [Jones and Peterlin, 1994; Caputo et al., 1995; Chang et al., 1995]. Because Tat is essential for virus replication and disease progression and is highly conserved among viral isolates [Myers et al., 1995], inhibition of Tat represents a very promising approach, both therapeutically and prophylactically, allowing inhibition of HIV replication and of the pathogenic effects associated to this protein. Thus, the development of an anti-Tat gene therapy system may possibly help control HIV infection.

Three Tat mutants in the transactivating domain (Tat_{22}, $Tat_{22/37}$, and Tat_{37}, cys_{22} to gly, cys_{37} to ser) were used previously to develop an experimental model

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for an anti-Tat gene therapy approach. These mutations abolish Tat transactivating activity and confer a transdominant negative phenotype [Balboni et al., 1993; Caputo et al., 1996]. Further experiments have shown that stable expression of Tat_{22} and $Tat_{22/37}$, but not Tat_{37}, interferes with replication and reactivation from latency of HIV-1 laboratory strains and primary isolates, both in human T-cell lines and peripheral blood lymphocytes from healthy donors and HIV-infected patients [Balboni et al., 1993; Caputo et al., 1996, 1997; Rossi et al., 1997]. However, the antiviral activity of the Tat_{22} and $Tat_{22/37}$ mutants is evident only against low doses of infectious virus in acute infection or against a low viral load in *in vivo* "chronically" infected cells [Caputo et al., 1996, 1997; Rossi et al., 1997].

To increase the activity of the Tat mutants, chimeric molecules were designed comprising the *tat* mutant genes fused to the Krüppel-associated box (KRAB) [Pengue et al., 1995]. Several studies indicate that the KRAB, a 75-aa long protein domain present in the amino termini of a large number of Krüppel-type Cys_2His_2 zinc finger proteins and evolutionarily conserved from yeast to humans, represents the strongest transcriptional repressor of gene expression identified so far in mammalian organisms [Bellefroid et al., 1991; Margolin et al., 1994; Pengue et al., 1994; Witzgall et al., 1994]. Transient transfection experiments in cell cultures have indicated that the KRAB domain fused to a heterologous DNA-binding protein can be targeted specifically to a DNA promoter or to a promoter-proximal RNA sequence. Under these conditions, KRAB acts as a strong transcriptional silencer of RNA polymerase II and III promoters, via a protein–protein interaction with some components of the transcriptional initiation complexes [Pengue and Lania, 1996; Moosmann et al., 1997]. These studies have led to the development of a novel general concept of intracellular immunization based on the use of artificial transcription factors containing a repressor domain fused to a DNA- or RNA-binding domain as therapeutic proteins for selected modulation of cellular or viral gene expression [Margolin et al., 1994; Pomerantz et al., 1995; Thiesen, 1996]. Thus, to increase the activity of the Tat mutants, a synergistic approach was attempted by constructing chimeric molecules comprising the tat_{22}, tat_{37}, or $tat_{22/37}$ genes fused to KRAB [Pengue et al., 1995]. These constructs combine a Tat transdominant negative mutant, with a silencer of gene expression, which is delivered specifically in the proximity of the HIV-1 long terminal repeat (LTR) promoter by the Tat mutant. It was shown previously that these chimeric molecules suppress, in transient assays, both basal and Tat-mediated activity of HIV-1 LTR-CAT gene expression more efficiently than the Tat mutants alone [Pengue et al., 1995], suggesting that they could be used successfully to control HIV-1 infection. Based on these encouraging results and similar observations reported by others [Margolin et al., 1994; Thiesen, 1996; Rauscher, 1998], in this study we investigated the use of the chimeric Tat mutant-KRAB fusion genes to control HIV-1 replication in acutely infected cells.

MATERIALS AND METHODS

Vectors

The cDNA (258 bp) of tat_{22}, tat_{37}, or $tat_{22/37}$, encoding an 86-aa protein, fused to the *KRAB* (172 bp) sequence [Gonsky et al., 1997], encoding a 57-aa protein, were amplified by polymerase chain reaction (PCR) from plasmids $pSVTat_{22}KRAB$, $pSVTat_{22/37}KRAB$, or $pSVTat_{37}KRAB$ described previously [Pengue et al., 1995], using primers 5′CATGGAGCCAGTAAGAC3′ (forward) and 5′TCTCGGTCATGGTCTC3′ (reverse). Amplification was carried out at 95°C (30 sec), 50°C (30 sec), 72°C (30 sec) for 35 cycles. The PCR products were cloned into the EcoRI and XhoI sites, filled in previously with Klenow, of the LXSN retroviral vector [Miller and Rosman, 1989]. Sequence analysis of the recombinant vectors was carried out by the chain termination reaction using the Sequenase version 2.0 kit (USB, Cleveland, OH). The reporter vector pU3RCAT, where the chloramphenicol-acetyl-transferase (CAT) gene is under the transcriptional control of the HIV-1 LTR promoter, was described previously [Sodroski et al., 1985]. Plasmid RSV-βgal, where the β-galactosidase reporter gene is expressed by the Rous sarcoma virus LTR promoter, was described previously [Norton and Coffin, 1985].

Cells

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum in atmosphere of 5% CO_2. Monolayer cultures of PA317 amphotropic packaging cells were cultured in DMEM supplemented with 10% heat inactivated Hyclone serum (Hyclone Laboratories, Logan, UT) and 1× HAT components (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) (Gibco) in atmosphere of 10% CO_2 and used to produce recombinant retroviruses, as described previously [Caputo et al., 1996]. The Jurkat T-cell line, derived from a human T-cell lymphoma, was grown in RPMI 1640 medium containing 10% heat inactivated fetal bovine serum (Gibco). Monoclonal and polyclonal cultures of Jurkat cells stably transduced with the recombinant retroviruses were selected in G418 (500 μg/ml) (Boehringer, Mannheim, Germany), as described previously [Caputo et al., 1996]. HeLa cells and the J/Ltatm-KRAB polyclonal cultures were transfected transiently by the DEAE-dextran method [Davis et al., 1986]. CAT assays were carried out and normalized for β-galactosidase activity [Caputo et al., 1996; Pengue et al., 1995].

Characterization of Jurkat Cell Lines Constitutively Expressing Tatm-KRAB Molecules

Cell surface CD4 expression was monitored in all J/Ltatm-KRAB cultures by flow cytometry, using an anti-CD4 monoclonal antibody [Balboni et al., 1993]. A

growth curve of each polyclonal culture was carried out by seeding 5×10^5 cells/ml in a total volume of 10 ml of complete medium containing G418. Viable cells were counted by Trypan blue exclusion at days 2, 4, 8, and 10. At each time point, concentration of the cells was readjusted to 5×10^5 cells/ml in a final volume of 10 ml. The growth patterns were compared by analysis of the variance for repeated measurements, considering the cell counts at each time point as dependent variable. Pairwise comparison of the cell counts was undertaken by the Bonferroni multiple comparison procedure [Snedecor and Cochran, 1968].

Extraction of total DNA and cytoplasmic RNA from cell cultures was carried out according to standard procedures [Davis et al., 1986]. Southern and Northern blot analysis [Davis et al., 1986] were carried out using nylon membranes (Hybond-N+; Amersham, Bucks, UK). *neo* DNA probes were ^{32}P-labeled by the random primer method, using the Multiprimer DNA labeling kit provided by Boehringer. Probe concentrations of 1×10^6 cpm/ml were used for hybridization reactions. Amplification of the *KRAB* sequences was carried out on 1 μg of total cellular DNA of J/Ltat$_{22}$KRAB, J/Ltat$_{22/37}$KRAB and J/Ltat$_{37}$KRAB cultures, using primers 5′GAAGCATCCAGGAAGTCAGCC3′ (forward), mapping at nucleotides 5862–5882 of the *tat* gene (HXBC2 clone), and 5′TTCGTCCAGATCATCCTGATCGAC3′ (reverse), mapping at nucleotides 630–607 of the *neo* sequence (Gene Bank, Locus ISTN5X). Amplification was carried out at 94°C (30 sec), 64°C (30 sec), 72°C (30 sec) for 35 cycles.

Expression of Tatm-KRAB chimeric proteins was assayed by flow cytometry. Briefly, exponentially growing cells (2×10^6) were harvested and permeabilized with 70% ethanol for 1 hr at 4°C. Cytometric detection of Tat molecules was conducted by immunofluorescence using a 1:100 dilution of a mouse anti-Tat monoclonal antibody (MAb; Intracell, London, UK), followed by incubation with a goat anti-mouse immunoglobulin G linked covalenty to fluorescein (GAM-F1; DAKO, Copenhagen, Denmark). Staining was performed in 200 μl phosphate-buffered saline containing 1% bovine serum albumin (BSA; Sigma), at 4°C for 30 min. Aspecific fluorescence was assessed with a nonspecific isotype-matched primary mouse MAb to cytomegalovirus (DuPont, Boston, MA) and GAM-F1 or with GAM-F1 alone. To quantify cell-associated fluorescence, before the assay the FACScan was calibrated by quantitative fluorescent microbeads (Flow Cytometry Standards Corporation). A standard curve was constructed by plotting the mean fluorescence intensity against the logarithm of the number of fluorescent molecules per bead. Samples were run in duplicate. Relative anti-Tat MAb binding sites per cell were obtained dividing the number of fluorescent molecules bound per cell by the fluorescence-to-protein ratio of the anti-Tat MAb. All experiments were carried out under conditions of saturation. The T53 cl.4 cell line, established from an adenocarcinoma of skin adnexa of BKV/*tat* transgenic mice, expressing high number of Tat molecules per cell, was described previously [Corallini et al., 1996] and was used as a control.

HIV-1 Infection

Virus stocks of HIV-1 were produced and titrated as described previously [Caputo et al., 1996]. The HXBC2 stock used in these experiments had a titer of 2×10^5 TCID$_{50}$/ml. Cells (3×10^6) were incubated in 1 ml of culture medium containing 3 μg of polybrene for 1 hr at 37°C. HIV-1 was then added at the multiplicity of infection (m.o.i). of 1 infectious particle (IP) per 1,000, 5,000, 10,000, or 50,000 cells. Cells were incubated for 3 hr at 37°C, washed twice, and resuspended in 6 ml of culture medium. Twice a week cells were counted and split by seeding 5×10^5 cells/ml in 6 ml. At the same time, 1 ml of culture supernatant was collected to assay for reverse transcriptase (RT) activity using an RT-enzyme-linked immunosorbent assay (ELISA) kit provided by Boehringer.

RESULTS

Retroviral vectors were constructed by inserting the Tat mutant-KRAB genes into the LXSN plasmid vector (Ltatm-KRABSN) (Fig. 1A) and ability to affect HIV-1 LTR-driven gene expression was examined by transient transfection assays. It was found that overexpression of the Ltatm-KRABSN vectors repressed both basal (Fig. 2A) and Tat-mediated (Fig. 2B) transcription of the reporter plasmid pU3RCAT, where expression of the chloramphenicol-acetyl-transferase reporter gene (CAT) is driven by the HIV-1 LTR. These results confirm previous experiments in which the Tat mutant-KRAB genes were expressed by a different plasmid under the transcriptional control of the SV40 early region promoter [Pengue et al., 1995]. Next, recombinant retroviruses were produced, after transient transfection of PA317 amphotropic packaging cell lines with the Ltatm-KRABSN vectors, and used to transduce the Jurkat T lymphocytic cell line, as described previously [Caputo et al., 1996]. Polyclonal mass cultures (m.c.) and monoclonal (cl.) cultures (J/Ltat$_{22}$-KRAB m.c., cl.1, cl.2, cl.4, cl.6, cl.7; J/Ltat$_{37}$-KRAB m.c., cl.4, cl.5, cl.6, cl.7, cl.8; J/Ltat$_{22/37}$-KRAB m.c. cl. 5, cl.7, cl.9, cl.11, cl.13), as well as control cultures transformed by the LXSN vector alone (J/LXSN m.c.) [Caputo et al., 1996], were selected in presence of G418 and characterized.

Cells transduced with Tat mutant-KRAB chimeric molecules were not affected in growth and proliferation as compared with control cultures, represented by Jurkat cells or by Jurkat cells transformed with LXSN, Ltat$_{22}$SN, Ltat$_{37}$SN, or Ltat$_{22/37}$SN vectors alone (J/LXSN m.c., J/Ltat$_{22}$SN m.c., J/Ltat$_{37}$SN m.c., J/Ltat$_{22/37}$SN m.c.) (Fig. 3). All cell lines exhibited cell surface CD4 molecules ranging from 55% to 98% of the cells as monitored by flow cytometry using a specific anti-CD4 monoclonal antibody (Table I). The integration pattern of the proviruses was determined on total cellular DNA digested with *SpeI* and *XbaI,* which cut respectively in the 5′ and 3′ LTRs of the vectors (Fig. 1A), allowing determination of the size of the integrated provirus.

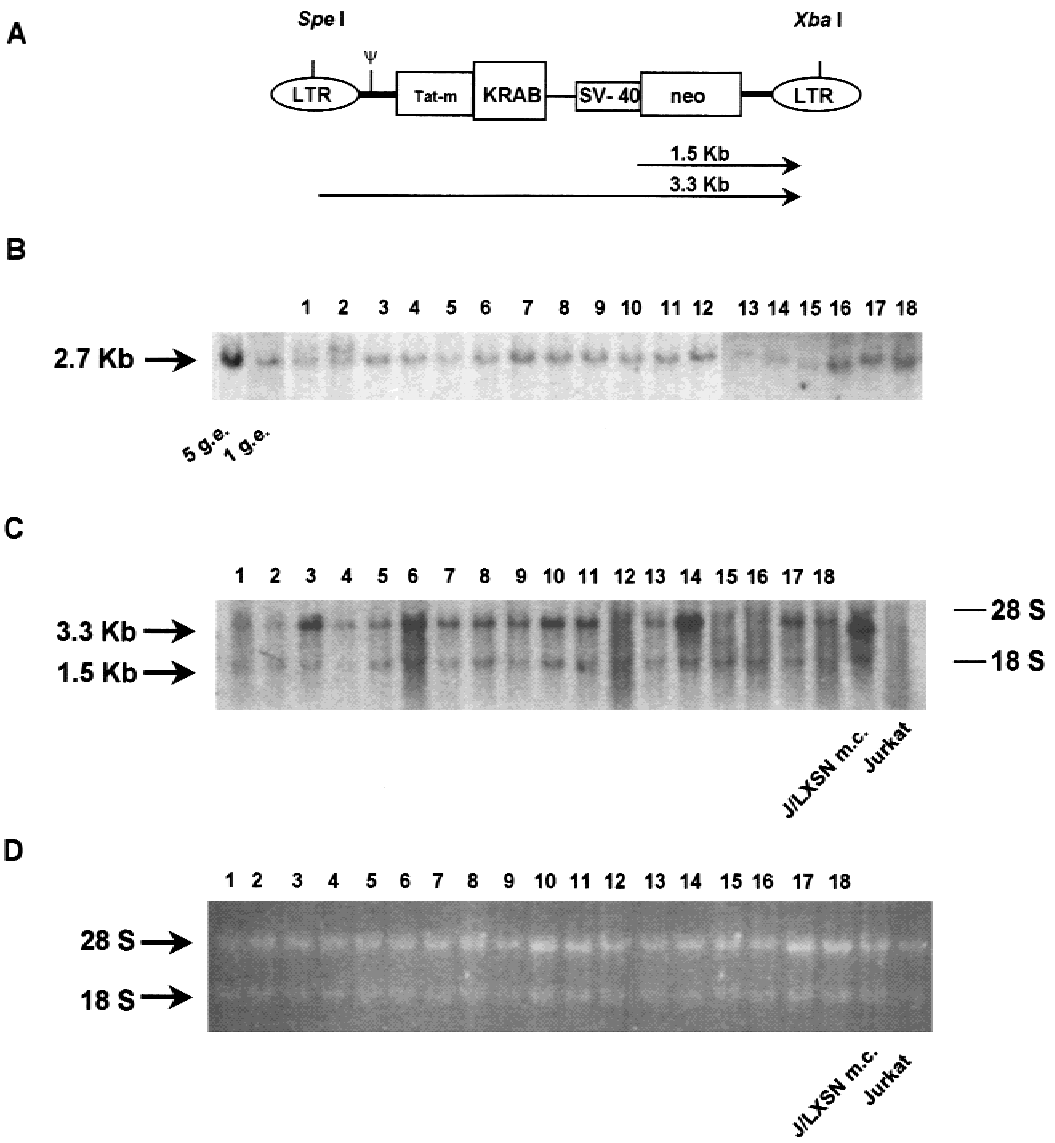

Fig. 1. Schematic representation of recombinant retroviral vectors Ltat$_{22}$KRABSN, Ltat$_{22/37}$KRABSN, and Ltat$_{37}$KRABSN, containing the *tat$_{22}$*, *tat$_{37}$*, or *tat$_{22/37}$* genes fused in frame to the DNA sequence encoding the KRAB repressor domain, and molecular characterization of polyclonal and monoclonal Jurkat cell lines J/Ltat$_{22}$KRAB, J/Ltat$_{22/37}$KRAB, and J/Ltat$_{37}$KRAB. **A:** Schematic representation of vectors. 5′ and 3′ long terminal repeats (LTRs) correspond to MoMLV and MoMSV, respectively. Ψ: packaging sequences. tat-m: cDNAs of *tat$_{22}$*, *tat$_{22/37}$*, and *tat$_{37}$* mutants. KRAB: Krüppel-associated box. neo: selectable gene under the transcriptional control of the SV40 early region promoter (SV40). Arrows mark the genomic (3.3 kb) and the neo (1.5 kb) mRNAs. **B:** Total cellular DNAs (10 μg) from J/Ltat$_{22}$KRAB, J/Ltat$_{22/37}$KRAB, and J/Ltat$_{37}$KRAB cultures were digested with *SpeI* and *XbaI* and analyzed by Southern blot hybridization to a neo-specific probe. One and five genome equivalents (g.e.) of Ltat$_{22}$KRABSN plasmid DNA, digested with *SpeI* and *XbaI,* was run as control. Lanes 1–6: J/Ltat$_{22}$KRAB clones 1, 2, 4, 6, 7, and mass culture (m.c.); lanes 7–12: J/Ltat$_{22/37}$KRAB clones 4, 5, 6, 7, 8, and m.c.; lanes 13–18: J/Ltat$_{37}$KRAB clones 5, 7, 9, 11, 13, and m.c. Arrow marks the 2.7-kb proviral DNA band. **C:** Northern blot analysis of cytoplasmic RNAs (20 μg). A neo-specific probe was used for hybridization. Lanes 1–6: J/Ltat$_{22}$KRAB clones 1, 2, 4, 6, 7, and m.c.; lanes 7–12: J/Ltat$_{22/37}$KRAB clones 4, 5, 6, 7, 8, and m.c.; lanes 13–18: J/Ltat$_{37}$KRAB clones 5, 7, 9, 11, 13, and m.c.; lane 19: J/LXSN m.c.; lane 20: normal Jurkat cells. Arrows point to the 3.3- and 1.5-kb mRNAs directed from the 5′LTR and the SV40 promoter. **D:** Ethidium bromide staining of the same formaldehyde-agarose gel, shown in (C), before Northern blot analysis. Arrows point to 28S and 18S ribosomal RNAs.

Hybridization with a *neo*-specific probe showed the presence of the expected 2.7-kb band in all monoclonal and polyclonal cultures, except for J/Ltat$_{37}$-KRAB cl.9 and cl.11, in which bands of smaller size were detected, suggesting integration of rearranged proviruses in these cell clones (Fig. 1B, lanes 15 and 16). Expression of the exogenous genes from the integrated proviruses was analyzed next by Northern blotting of cytoplasmic RNAs. Hybridization to the *neo* gene revealed the presence of the specific transcripts of 3.3 and 1.5 kb (Fig. 1C), directed from the Moloney murine leukemia virus long terminal repeat (MoMLV-LTR) and the SV40 promoter (Fig. 1A), in all monoclonal and polyclonal cell lines. These results indicate that the Tat mutant-KRAB sequences are expressed, except in J/Ltat$_{37}$-KRAB cl.9 and cl.11, in which the 3.3-kb transcript was not detected (Fig. 1C, lanes 15 and 16). The best clones (J/Ltat$_{22}$-KRAB m.c., cl. 2, cl. 4, cl. 7; J/Ltat$_{22/37}$-KRAB

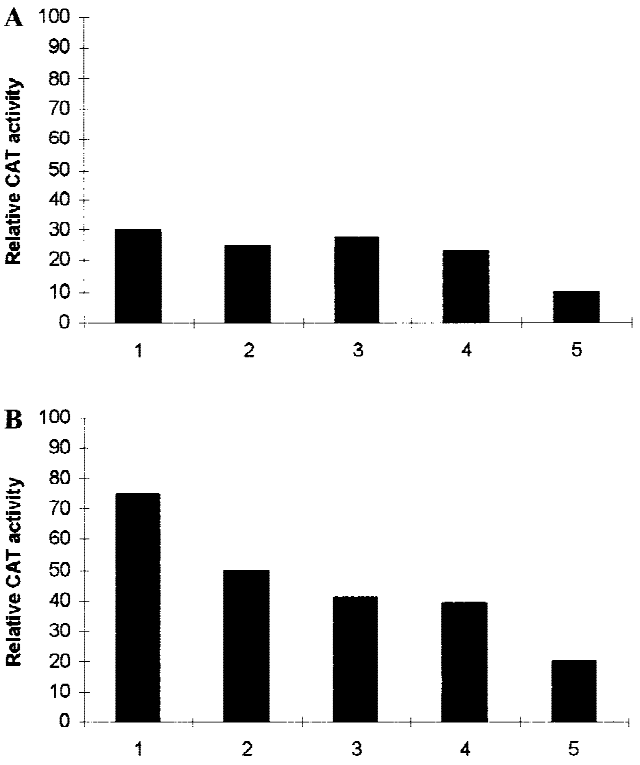

Fig. 2. **A:** The Krüppel-associated box (KRAB) domain represses basal HIV-1 long terminal repeat (LTR) promoter activity. HeLa cells were transfected with the reporter vector pU3RCAT (1μg) alone (lane 1) or co-transfected with pU3RCAT and each of the Ltat mutant vectors, 4 μg (lane 2) or 12 μg (lane 3), or co-transfected with pU3RCAT and each of the Ltatm-KRAB plasmids, 4 μg (lane 4) or 12 μg (lane 5). Chloramphenicol-acetyl-transferase (CAT) assays were performed on 200 μg of cell extract for 2 hr. **B:** Inhibitory effect of Tatm-KRAB on Tat-mediated HIV-LTR transactivation. HeLa cells were co-transfected with pU3RCAT (1μg) and pSV-Tat (1μg) (lane 1), or co-transfected with pU3RCAT, pSV-Tat, and each of the Ltat mutant vectors, 4 μg (lane 2) or 12 μg (lane 3), or co-transfected with pU3RCAT, pSV-Tat, and each of the Ltatm-KRAB plasmids, 4 μg (lane 4) or 12 μg (lane 5). CAT assays were performed on 100 μg of cell extract for 1 hr. In (A) and (B), cells were also transfected with a β-galactosidase reporter vector (2 μg) and CAT assays were normalized for the internal control β-galactosidase activity. In (A) and (B), one representative experiment with $Ltat_{22/37}$ and $Ltat_{22/37}$-KRAB is shown.

m.c., cl. 5, cl. 7, cl. 8, and J/$Ltat_{37}$-KRAB m.c., cl. 5, cl. 7, cl. 13) in terms of integration pattern of the proviral DNA and RNA expression of the chimeric genes were then tested for expression of the fusion proteins by flow cytometry, using an anti-Tat monoclonal antibody. As shown in Table II, all cell clones and mass cultures expressed a high number of chimeric molecules ranging from 950 to 10,800 per cell. Only J/$Ltat_{37}$-KRAB mass culture did not show detectable levels of the "therapeutic" gene.

The inhibitory activity of stably integrated Tat mutant-KRAB molecules on HIV-1 LTR-driven gene expression was first tested by transient transfection assays. As shown in Figure 4, constitutive expression of Ltatm-KRAB vectors repressed both basal and Tat-mediated transcription of the reporter pU3RCAT vector confirming the previous results. The inhibitory activity of the Tat mutant-KRAB molecules on HIV-1 replication was then tested on cells infected with HIV-1 at the m.o.i. of 1 IP per 1,000 cells, against which Tat_{22}, $Tat_{22/37}$, and Tat_{37} had not shown protection in our previous experiments [Caputo et al., 1996, 1997; Rossi et al., 1997]. Therefore, this dose of virus was the most suitable to show a synergistic effect of KRAB with the Tat mutants. Virus replication was monitored by assaying the RT activity released in the culture supernatants. These experiments showed that the chimeric genes, like the Tat mutants alone, did not exert a significant antiviral activity and did not exhibit a synergistic effect (Fig. 5A–C). Similar results were also observed when lower m.o.i. of 1 IP per 5,000 cells (Fig. 5D), and 1 IP per 10,000 or 50,000 cells were used (data not shown). These results were somehow unexpected, considering the transient assays that showed up to 99% inhibition of Tat transactivation on HIV-1 LTR by the combination of the transdominant *tat* and *KRAB* genes, as compared with an average inhibition of 40–50% caused by the Tat mutants alone [Pengue et al., 1995; Caputo et al., 1996]. Because KRAB is a potent transcriptional silencer, we reasoned that KRAB could have inhibited transcription of some vital cellular gene so that only cells containing an altered *KRAB* DNA could be selected for growth. We therefore amplified by PCR the *KRAB* sequences in the polyclonal cultures transformed by *tat_{22}-KRAB, $tat_{22/37}$-KRAB,* and *tat_{37}-KRAB*. Sequence analysis of the amplified products (data not shown) demonstrated that the nucleotide sequence of *KRAB* was identical to the reference sequence [Gonsky et al., 1997]. Therefore, the lack of HIV-1 inhibition cannot be attributed to KRAB toxicity leading to selection of cells containing a mutated *KRAB*.

DISCUSSION

Clearly, the results of the transient transfection experiments with Tat mutant-KRAB effectors and the HIV-1 LTR-CAT reporter, and the results obtained with stable integrated HIV-1 provirus and Tat mutant-KRAB chimeric proteins are not easily reconcilable. The most likely explanation for the discrepancy between the results of the transient assays and the effect of constitutive expression of KRAB on HIV-1 infection is that the structure of the target molecules (transiently transfected HIV-1 LTR-CAT reporter versus HIV-1 integrated genome) are different in the two types of experimental systems. Why should the Tat mutant-KRAB be sufficient for silencing HIV LTR-driven gene expression when templates are transfected transiently but not when the HIV LTR sequences are integrated into cellular DNA? In principle, this finding might arise from differential nuclear location of the two types of templates, or might reflect differences in chromatin structure. However, we favor the view that the inability of the Tat mutant-KRAB to repress integrated HIV-1 LTR is due to the chromosomal structure, so that Tatm-KRAB molecules do not have access to HIV-1 LTR. Indeed, a large body of evidence exists indicating that the chromosomal structure profoundly af-

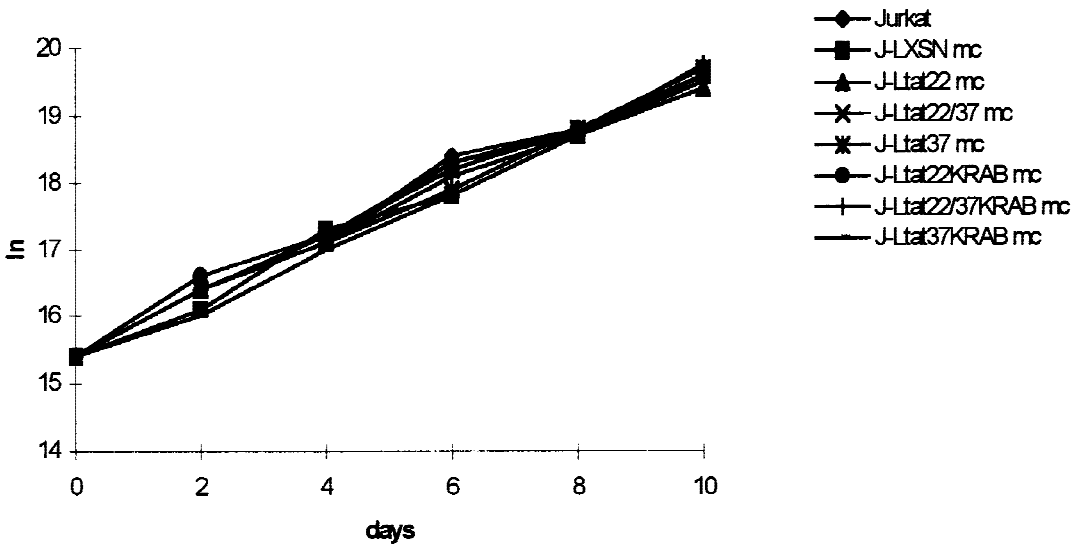

Fig. 3. Growth curve of Jurkat cell lines J/Ltat$_{22}$KRAB m.c., J/Ltat$_{22/37}$KRAB m.c., and J/Ltat$_{37}$KRAB m.c. expressing the Tatm-KRAB molecules, compared with Jurkat cells containing *tat*$_{22}$, *tat*$_{22/37}$, or *tat*$_{37}$ alone (J/Ltat$_{22}$ m.c., J/Ltat$_{22/37}$ m.c., J/Ltat$_{37}$ m.c.) or not expressing the "therapeutic genes" (J/LXSN m.c. and normal Jurkat cells). The data represent the average of two different experiments. Cell growth is represented in a semilogarithmic scale where the natural log (ln) of the cell number is indicated on the *y* axis.

TABLE I. CD4 Expression of Jurkat T Cells Transduced With Ltatm-KRABSN Recombinant Retroviruses

Cell line	Percentage of CD4$^+$
J/Ltat$_{22}$KRAB m.c.[a]	74
J/Ltat$_{22}$KRAB cl.2[b]	76
J/Ltat$_{22}$KRAB cl.4	80
J/Ltat$_{22}$KRAB cl.7	73
J/Ltat$_{22}$ m.c.	78
J/Ltat$_{22/37}$KRAB m.c.	67
J/Ltat$_{22/37}$KRAB cl.5	68
J/Ltat$_{22/37}$KRAB cl.7	86
J/Ltat$_{22/37}$KRAB cl. 8	68
J/Ltat$_{22/37}$ m.c.	98
J/Ltat$_{37}$KRAB m.c.	72
J/Ltat$_{37}$KRAB cl.5	80
J/Ltat$_{37}$KRAB cl.7	87
J/Ltat$_{37}$KRAB cl.13	80
J/Ltat$_{37}$ m.c.	55
J/LXSN m.c.	71
Jurkat	98

Cell surface CD4 expression was monitored by flow cytometry, using an anti-CD4 monoclonal antibody, in cell cultures transduced with the Ltatm-KRAB vectors, as well as in normal Jurkat cells or in Jurkat cells stably transduced by vectors LXSN (J/LXSN), Ltat$_{22}$SN (J/Ltat$_{22}$SN), Ltat$_{22/37}$SN (J/Ltat$_{22/37}$), or Ltat$_{37}$SN (J/Ltat$_{37}$), as described previously [Caputo et al., 1996].
[a]m.c., polyclonal culture; [b]cl., cellular clone.

TABLE II. Expression of Tatm-KRAB in Jurkat Cells Stably Transduced With Ltatm-KRAB Retroviruses

Cell lines	Tat molecules per cell[a]
J/Ltat$_{22}$KRAB m.c[b]	4,800
J/Ltat$_{22}$KRAB cl.2[c]	2,900
J/Ltat$_{22}$KRAB cl.4	2,900
J/Ltat$_{22}$KRAB cl.7	10,800
J/Ltat$_{22/37}$KRAB m.c.	9,800
J/Ltat$_{22/37}$KRAB cl.5	960
J/Ltat$_{22/37}$KRAB cl.7	4,900
J/Ltat$_{22/37}$KRAB cl.8	4,100
J/Ltat$_{37}$KRAB m.c.	Neg[e]
J/Ltat$_{37}$KRAB cl.5	1,400
J/Ltat$_{37}$KRAB cl.7	950
J/Ltat$_{37}$KRAB cl.13	4,800
J/LXSN m.c.	Neg.[e]
T53 cl.4[d]	5,350

[a]Number of Tat molecules per cell as determined by flow cytometry, using an anti-Tat monoclonal antibody; [b]m.c., mass culture; [c]cl., cellular clone; [d]T53 cl.4, included as control, expresses a high number of Tat molecules [Corallini et al., 1996]; [e]Neg., negative.

fects the transcription of the HIV-1 proviral genome [Verdin et al., 1993; el Kharroubi and Verdin, 1994; Van Lint et al., 1996; Sheridan et al., 1997]. Transcription of an integrated HIV-1 provirus in activated T cells and macrophages is accompanied by changes in chromatin structure at the LTR region [Verdin et al., 1993]. Moreover, specific inhibitors of histone deacetylase, such as trichostatin A (TSA), are potent inducers of HIV-1 transcription in latently infected T-cell lines and, most importantly, TSA treatment induces chromatin remodeling events at the LTR sequences that are the same as those observed in activated T cells [Van Lint et al., 1996]. These studies demonstrate clearly that chromatin remodeling is an important step for transcription of HIV-1 provirus, and they suggest that the level of histone H4 acetylation/deacetylation influences strongly proviral LTR-driven transcription. Remarkably, in a recent report it has been shown that HIV-1 transcription on chromatin template supports extremely high levels of transcription reinitiation as compared with naked DNA [Sheridan et al., 1997]. It has been proposed that chromatin remodeling induces a highly stable transcription complex that is able to perform multiple rounds of transcription initiation. On the other hand, several studies have demonstrated that transiently transfected DNA is assembled into nucleosome-like structures that exhibit abnormal repeat lengths and salt solubility [Jeong and Stein,

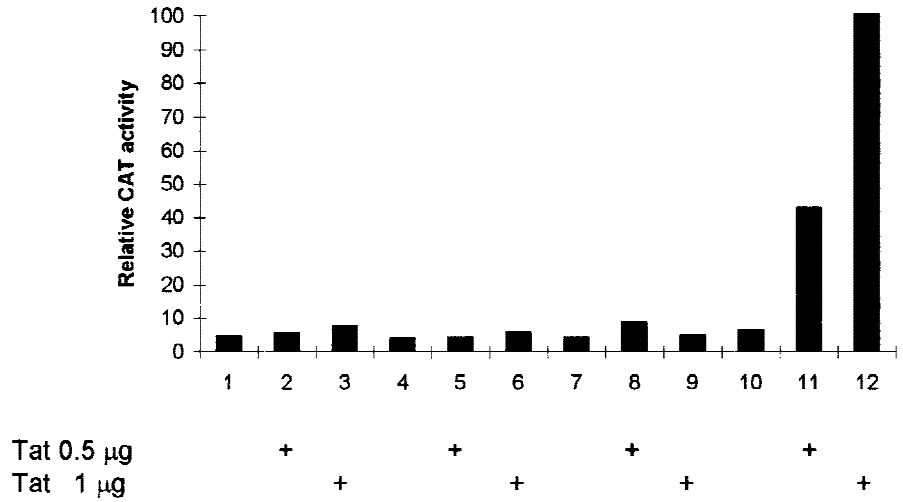

Fig. 4. Inhibitory effect of Tatm-KRAB on basal and Tat-mediated HIV-1 LTR transactivation. Jurkat cultures stably transduced by $Ltat_{22}$KRABSN (J/$Ltat_{22}$KRAB m.c., lanes 1–3), $Ltat_{22/37}$KRABSN (J/$Ltat_{22/37}$KRAB m.c., lanes 4–6), and $Ltat_{37}$KRABSN (J/$Ltat_{37}$KRAB m.c., lanes 7–9) recombinant retroviruses, and normal Jurkat cells (lanes 10–12), were transiently transfected with the reporter vector pU3RCAT (1 μg) alone, or co-transfected with pU3RCAT (1 μg) and 0.5 μg or 1 μg of pRP-Tat plasmid [Balboni et al., 1993]. CAT assays were performed on 200 μg of cell extract for 1 hr. Cells were also co-transfected with a β-galactosidase reporter vector (2 μg) and CAT assays were normalized for the internal control β-galactosidase activity.

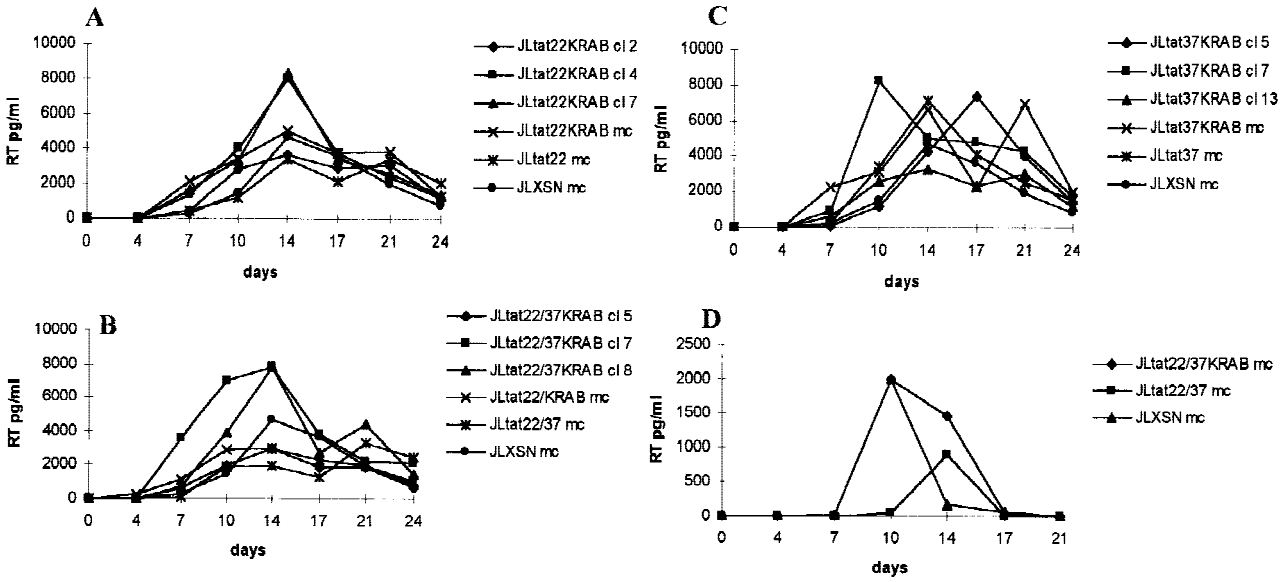

Fig. 5. Susceptibility to HIV-1 infection of Jurkat cultures stably transduced by $Ltat_{22}$KRABSN, $Ltat_{22/37}$KRABSN, and $Ltat_{37}$KRABSN recombinant retroviruses. Cells were infected at the multiplicity of infection (m.o.i.) of 1 infectious particle (IP) per 1,000 **(A–C)** or 5,000 **(D)** cells. At each time point cells were counted, split, and 1 ml of culture supernatant was collected to assay for reverse transcriptase (RT) activity. In (D), only one representative experiment is shown.

1994a, 1994b; Paranjape et al., 1994]. Moreover, it has been recently shown that transiently transfected and stably integrated DNAs respond differently to histone deacetylase inhibitors, which affect chromatin organization [Alberts et al., 1998]. Taken together, these recent studies demonstrate clearly that transcription on naked or abnormal chromatin-assembled HIV-1 LTR is dramatically different from HIV-LTR transcription of a provirus inserted in the chromatin structure [Sheridan et al., 1997].

The molecular mechanisms underlying the repressor ability of the KRAB domain are not known. It has been reported that KRAB is associated with a co-repressor factor named TIF1b [Friedman et al., 1996; Kim et al., 1996; Le Douarin et al., 1996; Moosmann et al., 1996]. However, so far no clues exist regarding the possible transcription partner of KRAB and TIF1b. Moreover, in a recent report it has been shown that KRAB-mediated repression is insensitive to TSA treatment, suggesting that KRAB-mediated repression does not involve histone deacetylation [De Luca et al., 1998]. Consequently, the activity of the proviral HIV-LTR

promoter, which is sensitive to histone deacetylation, may well be resistant to the action of KRAB. Conversely, overexpression of the KRAB domain tethered to HIV-1 LTR may well result in silencing of the HIV-1-driven transcription only in transient systems when the HIV-LTR sequences are not assembled into a correct nucleoprotein complex.

In conclusion, the results provide further evidence that transient assays may underestimate the contribution of the nucleoprotein structure to the requirement for transcriptional regulation, and they also indicate that careful caution must be used for applying directly results of transient assays for designing of gene therapy approaches for inhibition of HIV-1 infection.

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